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Glutamate Receptors Are Selectively Targeted to Postsynaptic Sites in Neurons

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Summary

The objective of the present study was to determine if a neuron that expresses multiple glutamate receptors targets the same receptors to all glutamatergic postsynaptic populations, or if the receptors are differentially targeted to specific postsynaptic populations. As a model for this study, we chose the fusiform cell of the dorsal cochlear nucleus. This neuron expresses multiple glutamate receptors and receives two distinct glutamatergic inputs: parallel fibers synapse on apical dendrites, and auditory nerve fibers synapse on basal dendrites. Pre- and postembedding immunocytochemistry were combined with retrograde tracing to identify the receptors expressed on postsynaptic membranes of parallel fiber and auditory nerve synapses. Most receptors were found at both populations of synapses, but the AMPA receptor subunit, GluR4, and the metabotropic receptor, mGluR1α, were found only at the auditory nerve synapse. These results demonstrate that glutamate receptors are targeted to specific postsynaptic populations of glutamatergic synapses.

Introduction

Molecular cloning studies have identified multiple glutamate receptors in mammalian brain. These include three ionotropic receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainate, and *N*-methyl-D-aspartate (NMDA), and eight metabotropic receptors (reviewed by Hollmann and Heinemann, 1994). Delta receptors are thought to represent a fourth type of ionotropic glutamate receptor, based on sequence similarity to other glutamate receptors, but the lack of a demonstrable function of both δ subunits (Araki et al., 1993; Lomeli et al., 1993) leaves their glutamate receptor status in doubt. With in situ hybridization and immunocytochemistry, it has been shown that most neurons in the mammalian central nervous system express multiple glutamate receptor subtypes and subunits (Hollmann and Heinemann, 1994; Petralia and Wenthold, 1996; Petralia, 1997). Physiological and immunocytochemical studies have found that two or more glutamate receptors can exist at the same postsynaptic structure (Bekkers and Stevens, 1989; Jones and Baughman, 1991; Hollmann et al., 1991; Verdoorn et al., 1991; Petralia and Wenthold, 1992; Nusser et al., 1994; Petralia et al., 1994a, 1994b; Isaac et al., 1995; Liao et al., 1995; Petralia, 1997; Bernard et al., 1997; Landsend et al., 1997). A fundamental question, therefore, concerns the mechanism by which the various glutamate receptors are distributed within a single neuron. All glutamatergic synapses on a neuron may have the same composition of postsynaptic receptor subunits and subtypes; alternatively, the neuron may have the capability to selectively target receptors to various synaptic populations. The second scenario would allow physiological responses to vary with the synaptic input, depending on the differential postsynaptic expression of receptors. Furthermore, the compositions of synaptic receptors could change at individual synaptic populations during conditions such as development or synaptic use. A number of biochemical and physiological studies have suggested a differential receptor distribution (Zalutsky and Nicoll, 1990; Ajima et al., 1991; Derrick et al., 1991; Lerma et al., 1994; Liao, 1995; Isaac et al., 1995; Wenthold et al., 1996), but there is little direct evidence to support such a model. On the other hand, selective targeting of presynaptic metabotropic receptors was recently demonstrated in the hippocampus (Shigemoto et al., 1996) and the retina (Brandstätter et al., 1996).

The objective of this study was to determine if a neuron that expresses multiple glutamate receptors targets the same receptors to all glutamatergic postsynaptic populations, or if the receptors are differentially targeted to specific postsynaptic populations. As a model neuron for this study, we chose the fusiform cell of the dorsal cochlear nucleus (DCN). The fusiform cell expresses multiple glutamate receptors, including AMPA, kainate, NMDA, δ, and metabotropic subtypes (Oertel and Wu, 1989; Hunter et al., 1993; Bilak et al., 1996; Petralia et al., 1996), and receives two distinct glutamatergic inputs (Manis, 1990; Juiz et al., 1993; Osen et al., 1995; Manis and Molitor, 1996; Hackney et al., 1996). The auditory nerve terminates on its basal dendrites (Lorente de No, 1981; reviewed by Cant, 1992; Ryugo and May, 1993), and the parallel fibers, originating from granule cells of the DCN, terminate on its apical dendrites (Kane, 1974; Mugnaini et al., 1980; Lorente de No, 1981; Smith and Rhode, 1985; Hackney et al., 1990). Although fusiform cell synapses cannot be readily differentiated from those of other neurons in the DCN based only on their morphological characteristics, fusiform neurons project to the contralateral inferior colliculus (Lorente de No, 1981; Ryugo and May, 1993), allowing them to be easily and specifically retrogradely labeled with tracers. In the present study, we have used pre- and postembedding immunocytochemistry, along with retrograde tracing, to characterize glutamate receptor expression at auditory nerve and parallel fiber synapses on fusiform cells. Our results support a model in which glutamate receptors are selectively targeted to specific postsynaptic populations.

Results

Identification of Fusiform Cell Synapses

To identify fusiform cells and their apical and basal dendrites, horseradish peroxidase (HRP) was injected into the inferior colliculus of rats and allowed to retrogradely

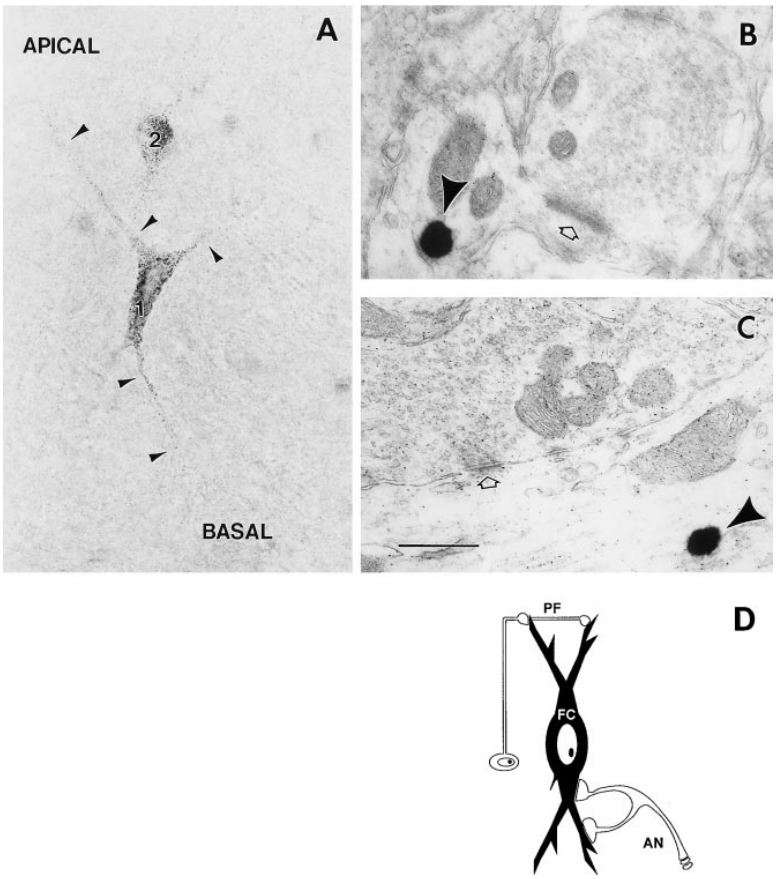

Figure 1. Fusiform Cells of the DCN Retrogradely Labeled with HRP after the Injection in the Inferior Colliculus

(A–C) At the light microscopic level (A), granules of HRP are seen in the soma (1 and 2) and in the apical and basal dendrites (arrowheads) of fusiform cells. At the ultrastructural level, the reaction product appears as electron dense organelles (arrowhead) in the cytoplasm in apical (B) and basal (C) dendrites. In (B), the HRP-positive organelle is close to a spine that makes synaptic contact with a parallel fiber; in (C), it is observed in a longitudinal section of a basal dendrite of the fusiform cell, with the auditory nerve terminal making synaptic contact on the dendrite. Postsynaptic densities are unlabeled (open arrows). Scale bar, 0.5 μm.

(D) A schematic drawing that shows the excitatory synaptic input on fusiform cells. AN, auditory nerve; FC, fusiform cell; PF, parallel fiber.

transport to the fusiform cell. The fusiform cell is the only neuron in this area of the DCN that projects to the inferior colliculus (Lorente de No, 1981; Ryugo and May, 1993). At the light microscopic level, the reaction product of HRP developed with 3′,3-diaminobenzidine (DAB) appeared as dark brown granules, labeling fusiform cell somata and extending into the apical and basal dendrites (Figure 1A). Electron microscope analysis showed electron dense organelles in the cytoplasm of fusiform cells; these organelles extended into the distal-most processes of the dendrites. The HRP reaction product was confined to the organelle and did not extend to other cellular elements (Figures 1B and 1C). Therefore, this approach could be used together with immunocytochemistry to identify the apical and basal dendrites of fusiform cells.

Excitatory synaptic inputs from parallel fibers are the predominant synaptic population on apical dendrites of

Table 1. Antibodies Used to Localize Glutamate Receptors

	Concentration		
Receptor	Preembedding	Postembedding	Reference
AMPA			
GluR2/3 (polyclonal)	1.0 μg/ml	2.5 μg/ml	Wenthold et al., 1992
GluR2/3 (monoclonal)	—	1:7500 dilution	Nusser et al., 1994; Ottiger et al., 1995
GluR2 (polyclonal)	1.5 μg/ml	4.0 μg/ml	Petralia et al., 1997
GluR4 (polyclonal)	1.5 μg/ml	2.0 μg/ml	Wenthold et al., 1992
NMDA			
NR1 (monoclonal)	1:1000 dilution	—	Sucher et al., 1993
NR1 (polyclonal)	2.0 μg/ml	4.0 μg/ml	Petralia et al., 1994a
NR2A/B (polyclonal)	1.5 μg/ml	4.0 μg/ml	Petralia et al., 1994b
Metabotropic			
mGluR1α (monoclonal)	1:300 dilution	1:10 dilution	Petralia et al., 1997
mGluR1α (polyclonal)	1.5–2.0 μg/ml	—	Petralia et al., 1996
Delta			
Delta1/2 (polyclonal)	1.5 μg/ml	2.0 μg/ml	Mayat et al., 1995

fusiform cells and the only excitatory input to these dendrites (Smith and Rhode, 1985; Oertel and Wu, 1989; Manis, 1990; Osen et al., 1995). Parallel fiber synapses were identified using criteria established by Kane (1974), Mugnaini et al. (1980), and Cant (1992), which include their location in the molecular layer of the DCN (parallel fibers are described as unmyelinated axons that run parallel to the surface of the nucleus) and their ultrastructural characteristics. The parallel fiber synapses are small synaptic endings that contain small, clear synaptic vesicles and make asymmetrical synaptic contacts (Gray I) onto spines and/or dendritic shafts of apical dendrites of fusiform cells.

Auditory nerve synapses are the predominant excitatory synapses on basal dendrites of fusiform cells (Cant and Morest, 1984; Smith and Rhode, 1985; Wickesburg and Oertel, 1989). These endings were identified using criteria established by Kane (1974), Smith and Rhode (1985), and Ryugo (1992), which include their location in the nucleus (the auditory nerve synapses are distributed in the fusiform and deep layers of the DCN) and their ultrastructural characteristics. The auditory nerve synapses are defined as large presynaptic endings (in the cat, $\sim$50–60 μm in diameter) that have numerous large, clear, and round synaptic vesicles (in the cat, 54.5 $\pm$ 4.4 nm, mean $\pm$ SD, in diameter) and contain numerous mitochondria. The synaptic contacts are asymmetric (Gray I) and are relatively small when compared to the total membrane apposition. Each ending, in addition to the short synaptic contact, is also characterized by attachment plaques or "puncta adherentia."

Preembedding Immunocytochemistry

Glutamate receptor distribution in fusiform cells was determined with both pre- and postembedding immunocytochemistry. Preembedding immunocytochemistry with HRP has the advantage of being more sensitive and is not affected by the embedding procedures; however, antibody binding cannot be readily quantified, and lack of penetration through the section can lead to false negatives (Nusser et al., 1994; Baude et al., 1995). Postembedding staining with immunogold allows quantitation, and labeling is not affected by penetration of antibody; however, it is less sensitive than preembedding HRP, and some antigenic sites appear not to tolerate the embedding procedure (Nusser et al., 1994; Baude et al., 1995). With their complementary strengths and weaknesses, both techniques were used in the present study.

Preembedding immunocytochemistry allowed simultaneous visualization of the retrogradely-transported organelle and receptor immunostaining, with both reaction products being readily distinguishable. For all antibodies used, staining was most intense at the postsynaptic densities, but was also found in the dendrite cytoplasm and associated with membranes of the smooth endoplasmic reticulum. However, as previously reported (Petralia and Wenthold, 1992; Petralia, 1997), the relative amount of the intracellular staining varied with the antibody. In analyzing receptor distribution, only apical and basal dendrites of fusiform cells, identified by the presence of an HRP-positive organelle either in the same section or in a consecutive ultrathin section, were considered and analyzed.

Examples of preembedding staining of receptors in fusiform cells are shown in Figures 2–4. These results are representative of 8–30 individual postsynaptic densities identified for each synaptic population (auditory nerve and parallel fiber synapses) for each antibody for a total of 260 synapses analyzed. All of the synapses analyzed were obtained from ultrathin sections 2–7 μm deep in the tissue. Postsynaptic densities were categorized as "labeled" or "unlabeled." A labeled postsynaptic density was defined as one with intense staining, clearly above background, and an unlabeled postsynaptic density as one with staining like that of background. To account for a possible lack of penetration of antibodies and reagents producing false negatives, postsynaptic densities were considered unlabeled only if labeled structures were present on the same section. Most of the auditory nerve and parallel fiber synapses could be classified in one or the other category. Antibodies to GluR2, GluR2/3, NR1, NR2A/B, and δ 1/2 routinely gave postsynaptic labeling of both auditory nerve and parallel fiber synapses. In contrast, antibodies to GluR4 and to mGluR1α only gave postsynaptic labeling of auditory nerve synapses. For mGluR1α, this pattern was seen using two different antibodies (not shown). While postsynaptic labeling of apical synapses was not seen with antibodies to GluR4 and mGluR1α, labeling was present on intracellular membranes in apical dendrites (Figure 2; GluR4). Furthermore, at the light microscopic level, labeling was present in the proximal segments of both apical and basal dendrites for all antibodies, including those to GluR4 and mGluR1α (data not shown; also see Petralia et al., 1996).

Postembedding Immunocytochemistry

Postembedding immunocytochemistry also allowed simultaneous visualization of the retrogradely-transported organelles and receptor immunostaining. A general distribution similar to that seen for preembedding immunocytochemistry was seen for all antibodies, with the most intense labeling at postsynaptic densities and with lighter labeling of intracellular membranes.

Examples of the postembedding immunogold labeling of fusiform cells are shown in Figures 5 and 6, and a summary of the quantitative analyses of labeling is shown in Table 2. Qualitatively, the results are like those obtained with preembedding staining, with GluR4 and mGluR1α being associated only with auditory nerve synapses (5 nm and 10 nm gold particles were used, obtaining the same results). Double labeling for GluR2/3 and GluR4, using 15 nm and 5 nm gold particles, respectively, confirmed that both subunits are present at the postsynaptic density of auditory nerve synapses, but only GluR2/3 is found postsynaptic of parallel fiber terminals (Figure 5). By quantitating immunogold labeling, it is seen that there is a four-fold greater concentration of δ 1/2 at parallel fiber synapses compared with auditory nerve synapses (Table 2). Labeling with antibodies to GluR2, GluR2/3, and NR2A/B showed a similar density of immunogold at both synaptic populations. Significant immunogold labeling ($>$2 gold particles per synapse) was not obtained with the monoclonal or polyclonal antibody to NR1 for either synaptic population.

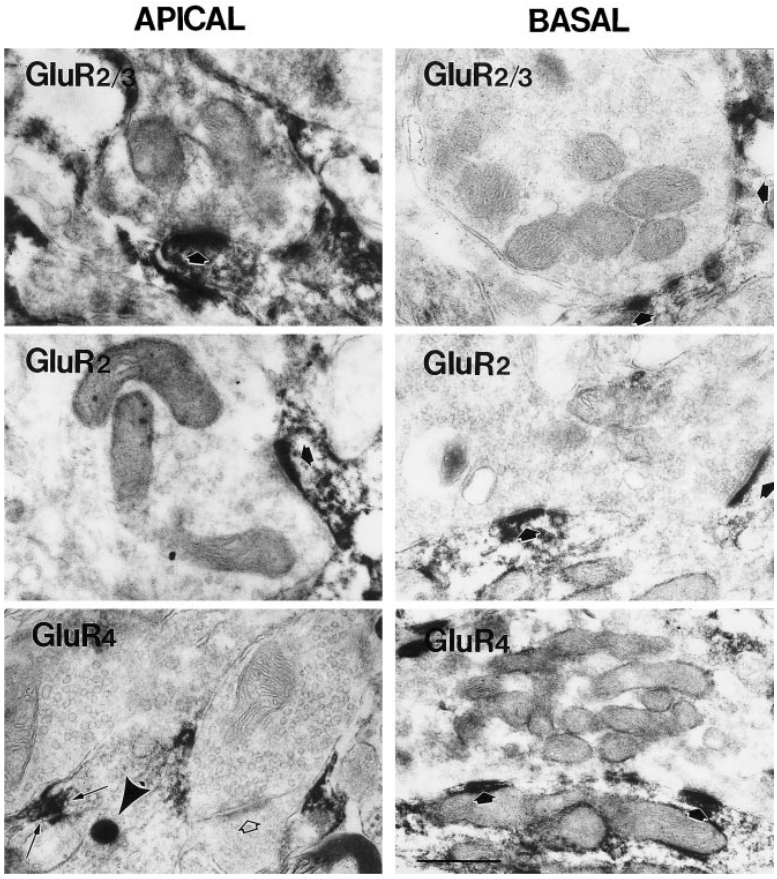

Figure 2. Preembedding Immunoreactive Labeling with HRP Using Antibodies Selective for AMPA Receptor Subunits GluR2/3, GluR2, and GluR4 in Apical and Basal Dendrites of Fusiform Cells

Postsynaptic membranes of parallel fiber synapses on apical dendrites were immunopositive for GluR2/3 and GluR2, but not for GluR4, while postsynaptic membranes of auditory nerve synapses on basal dendrites are immunoreactive for all three antibodies. An HRP-positive organelle (arrowhead) is seen in the cytoplasm of an apical dendrite after the immunolabeling for GluR4. The large, closed arrows show postsynaptic labeling, and the large, open arrows show unlabeled synapses. The small, closed arrows show cytoplasmic labeling (GluR4). Scale bar, 0.5 μm.

Discussion

The results of this study show that glutamate receptors are selectively targeted to postsynaptic populations in fusiform neurons. Two proteins, GluR4, an AMPA receptor subunit, and mGluR1α, a metabotropic glutamate receptor, are present at auditory nerve synapses on basal dendrites but absent from parallel fiber synapses on apical dendrites. While GluR4 and mGluR1α were not detected at parallel fiber synapses, some subunits were present at both synaptic populations, but at different levels; quantitating immunogold labeling shows that δ 1/2 is more than four-fold more abundant at parallel fiber synapses than at auditory nerve synapses. Staining with three other antibodies, selective for GluR2, GluR2/3, and NR2A/B, shows that these subunits are equally abundant at both synapses. The presence of GluR2 at both synapses indicates that the AMPA receptors formed are calcium impermeable. Qualitatively, similar results were obtained with both preembedding immunocytochemistry with HRP detection and postembedding immunocytochemistry with colloidal gold detection. An advantage of the preembedding/HRP method is its greater sensitivity, due to an enzymatic detection system; its ability to detect low levels of receptor is valuable in assessing possible false negative results obtained with immunogold. With both techniques, GluR4 and mGluR1α were not detected at parallel fiber synapses, while the other receptors were seen at both populations.

Functional Significance

A selective distribution of glutamate receptors endows a neuron with the capability of having functionally different receptors at different synaptic populations. Such a differential distribution would be consistent with the functional properties that are characteristic of these two synaptic populations on fusiform neurons. Electrophysiological experiments have identified NMDA receptors, but not metabotropic glutamate receptors, on apical dendrites of fusiform cells (Manis and Molitor, 1996; Molitor and Manis, 1997). Studies on the chick cochlear nucleus have shown that synapses formed between the auditory nerve and principal neurons in the cochlear nucleus contain AMPA receptors that have exceptionally fast desensitization and deactivation rates (Raman et al., 1994). This unusual speed does not appear to be restricted to the chick or to the cochlear nucleus, since similar results were found in the rat medial nucleus of the trapezoid body (MNTB), which receives synaptic input from the cochlear nucleus. Single cell PCR analysis of MNTB neurons showed a high level of GluR4 (Geiger et al., 1995), and a study on recombinant AMPA receptor subunits expressed in oocytes showed that AMPA receptors containing GluR4 are the most rapidly-desensitizing subunits (Mosbacher et al., 1994). Therefore, GluR4 may make AMPA receptors at the fusiform cell auditory nerve synapse with more rapid channel kinetics than those at the parallel fiber synapses, which do not contain GluR4, similar to AMPA receptors at other synapses

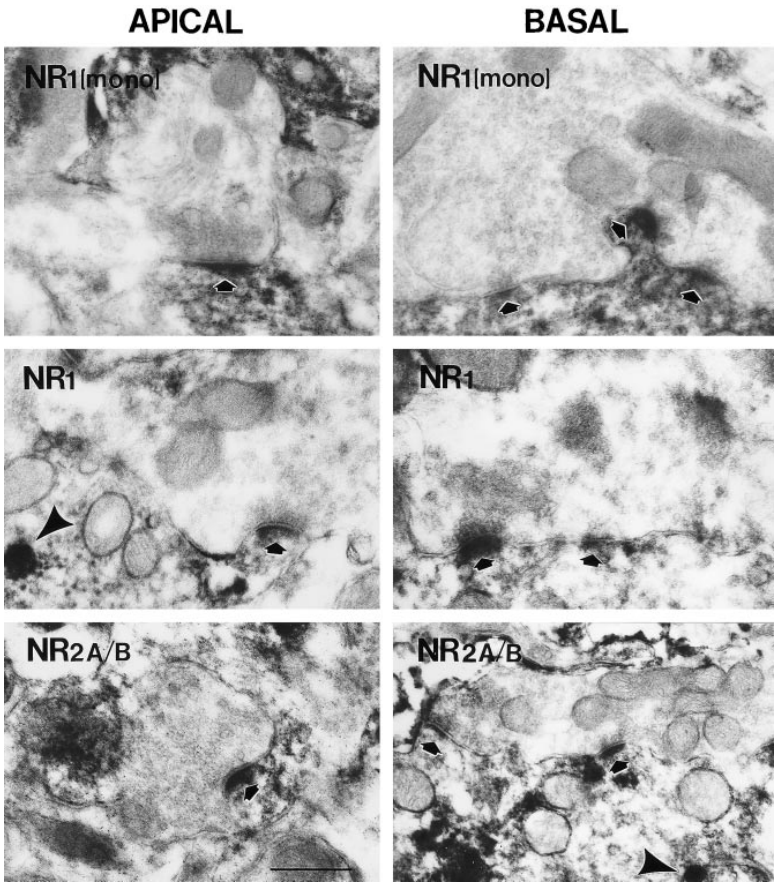

Figure 3. Preembedding Immunoreactive Labeling with HRP Using Antibodies Selective for NMDA Receptor Subunits NR1 (NR1[mono], Monoclonal; NR1, Polyclonal) and NR2A/B in Apical and Basal Dendrites of Fusiform Cells

The micrographs on the left show parallel fibers making synaptic contact on a dendrite shaft (NR1 mono) and on dendritic spines (NR1 polyclonal, NR2A/B) of apical dendrites of fusiform cells; the postsynaptic membranes (large, closed arrows) are labeled with antibodies to NR2A/B and both antibodies to NR1. The postsynaptic densities of basal dendrites apposed to auditory nerve terminals are also strongly labeled with all three antibodies. HRP-positive organelles (arrowheads) are seen in most cases. Scale bar, 0.5 μm.

along the ascending auditory pathway. This property would allow neurons in the cochlear nucleus and other brain stem nuclei to convey accurate temporal information to higher structures and to maintain interaural timing differences, which are critical for sound localization (Raman et al., 1994; Trussell et al., 1994).

Mechanisms of Selective Targeting

Selective targeting of a protein in a cell can be achieved by two mechanisms, targeting of the mRNA or targeting of the protein itself (Dotti and Simons, 1990; Matter et al., 1990; Hoop and Dotti, 1993; Keely and Grote, 1993; Steward, 1997). mRNAs have been shown to be differen-

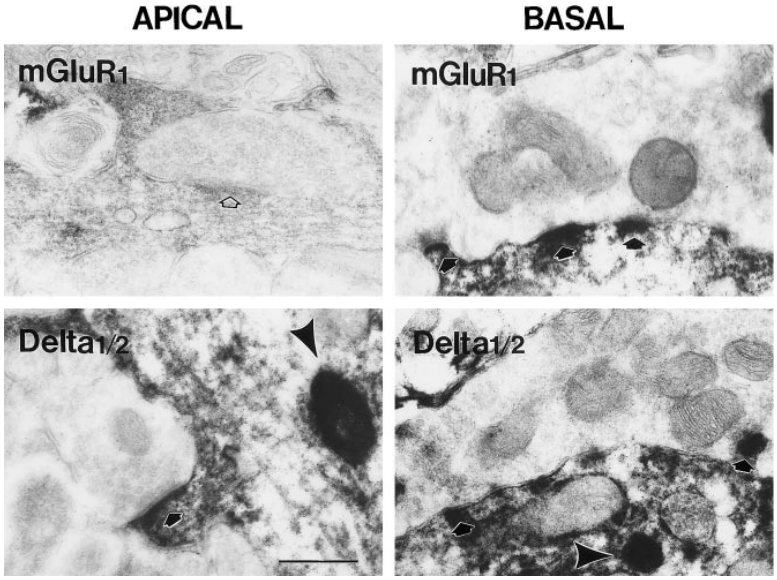

Figure 4. Preembedding Immunoreactive Labeling with HRP Using Antibodies Selective for mGluR1α (Polyclonal Antibody) and Delta 1/2 Subunits in Apical and Basal Dendrites of Fusiform Cells

The micrographs on the left show parallel fiber synapses onto an apical dendritic shaft (mGluR1α) and a spine (δ 1/2) of the fusiform cell. The postsynaptic membranes are labeled (large, closed arrows) with antibodies to δ 1/2 but unlabeled with antibodies to mGluR1α (large, open arrows). The postsynaptic membranes of basal dendrites of fusiform cells are labeled with antibodies to both δ 1/2 and mGluR1α (large, closed arrows). The arrowheads show HRP-labeled intracellular organelles. Scale bar, 0.5 μm.

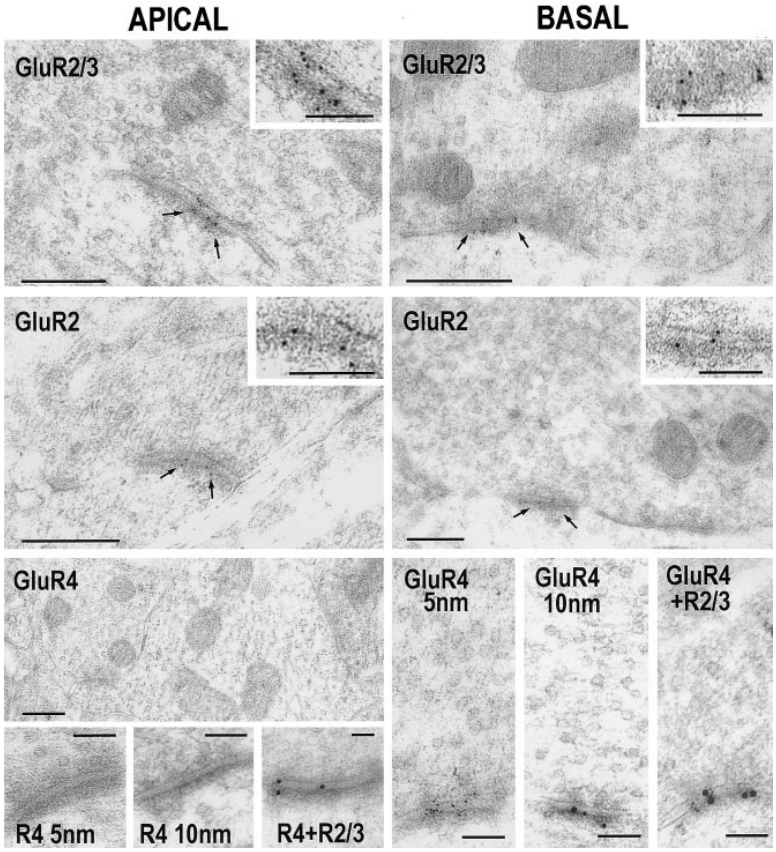

Figure 5. Postembedding Immunogold Labeling with Antibodies Selective for AMPA Receptor Subunits GluR2/3 (5 nm), GluR2 (5 nm Gold), and GluR4 (5 nm and 10 nm Gold) in Apical and Basal Dendrites of Fusiform Cells

On the postsynaptic membranes of parallel fiber synapses with apical dendrites (left panels), immunogold labeling is seen with antibodies to GluR2/3 (monoclonal antibody) and GluR2 but not with antibodies to GluR4. On the contrary, the postsynaptic membranes of auditory nerve synapses on basal dendrites (right panels) show immunogold labeling for all three antibodies. The insets of GluR2/3 and GluR2 show higher magnifications of the postsynaptic densities (small, closed arrows). The insets at the bottom left show the postsynaptic membranes of parallel fiber synapses at apical dendrites of fusiform cells after immunogold labeling for GluR4 using 5 nm and 10 nm gold, and double labeling for GluR4 (5 nm gold) and GluR2/3 (monoclonal antibody, 15 nm gold). In the latter case, only GluR2/3 labeling is present at the postsynaptic membranes of parallel fibers on apical dendrites. At the bottom right, the postsynaptic membranes of the auditory nerve are immunolabeled for GluR4 (5 nm, 10 nm gold), and double labeled for GluR4 (5 nm gold) and GluR2/3 (monoclonal, 15 nm gold). Scale bars for GluR2/3 and GluR2, 0.25 μm; for the insets, 0.1 μm; for GluR4 (low magnification), 0.5 μm; for the insets, 0.1 μm.

tially distributed in a range of cells, including neurons for which several studies have demonstrated the presence of mRNA in dendrites (Miyashiro et al., 1994; Tiedge and Brosius, 1996; Martone et al., 1996; Gazzaley et al., 1997; Racca et al., 1997). It was recently reported that the glycine receptor α subunit mRNA is present in both the somata and dendrites of spinal cord neurons, where it is near postsynaptic sites containing the glycine receptor (Racca et al., 1997). In contrast, the β subunit mRNA is predominantly in the somata. Several studies have documented the presence in dendrites of multiple endoplasmic reticulum and Golgi enzymes necessary for folding, assembly, and posttranslational modification (Villa et al., 1992; Volpe et al., 1993; Torre and Steward, 1996). In the fusiform neuron, selective expression of mRNA in the basal dendrites could account for the presence of GluR4 and mGluR1α at auditory nerve synapses but not at parallel fiber synapses. Present evidence, however, does not support a significant amount of glutamate receptor mRNA in dendrites, although this has not been thoroughly explored for all subunits. In cultured hippocampal neurons, AMPA receptor mRNAs are predominantly found in the cell body (Eshhar et al., 1993), and studies on tissue sections also show a heavy cell body labeling (Monyer et al., 1991; Sato et al., 1993; Lauri and Seeburg, 1994; Bahan and Wisden, 1997). NR1 mRNA is not normally present at detectable levels in dendrites, but has been shown to increase in hippocampal neurons after perforant path lesion (Gazzaley et al., 1997). In situ hybridization of AMPA receptor mRNA in the DCN of the normal adult rat showed that mRNA is concentrated in fusiform cell bodies, with no obvious dendritic concentration (Hunter et al., 1993).

Since most mRNA is found in neuronal cell bodies, targeting of the protein itself is likely to be the mechanism by which most proteins are targeted in neurons. A number of proteins have been shown to be selectively transported to axonal and somal/dendritic compartments (Dotti and Simons, 1990; Dotti et al., 1991; Huber et al., 1993; Kunimoto, 1995; Cid-Arregui et al., 1995; Terada et al., 1996). Sorting of membrane proteins destined for axons or dendrites probably occurs in the trans-Golgi network, and targeting may depend on signals on the intracellular membrane vesicles which recognize cytoskeletal differences, such as microtubule polarity, between axons and dendrites (Kelly and Grote, 1993). However, there is little information concerning the mechanism by which membrane proteins are selectively targeted to specific locations within dendrites. In nonneuronal cells, selective targeting of membrane proteins, e.g., to apical and baso-lateral poles of polarized epithelial cells, is accomplished by targeting domains on the protein (Wozniak and Limbird, 1996; Odorizzi et al., 1996; Yoshimori et al., 1996). Therefore, individual proteins, destined for a specific synaptic population, may be packaged together in a vesicle and sent directly to a postsynaptic location. Given the large number of different dendritic domains, an alternative mechanism, in which all dendritic proteins are packaged together, appears more feasible. In this model, the organelle would contain only

Table 2. Summary of the Postembedding Immunoreactivity for Glutamate Receptor Subunits at the Auditory Nerve and Parallel Fiber Synapses

	Auditory Nerve Synapses (Basal Dendrites)				Parallel Fiber Synapses (Apical Dendrites)			
Receptors	Number of PSDs	Average Length of PSD (μm)	Average Number of Gold Particles per PSD (Range)	Number of Gold Particles per μm of PSD ± SE	Number of PSDs	Average Length of PSD (μm)	Average Number of Gold Particles per PSDs (Range)	Numer of Gold Particles per μm of PSD ± SE
GluR2/3*†	18	0.25	3.6 (1–8)	17.7 ± 4.0	17	0.27	3.7 (1–8)	16.5 ± 3.2
GluR2†	25	0.22	1.9 (0–6)	9.1 ± 1.1	17	0.29	2.2 (0–4)	7.2 ± 1.2
GluR4†	17	0.24	4.5 (2–9)	19.1 ± 2.2	17	0.26	0	0
GluR4 (10 nm gold)	9	0.16	1.7 (0–5)	9.2 ± 1.9	8	0.30	0	0
NR2A/B†	19	0.25	1.5 (0–5)	6.4 ± 1.4	14	0.29	2.6 (0–4)	9.8 ± 1.3
mGluR1α*†	35	0.20	1.6 (0–4)	8.0 ± 1.3	25	0.29	0	0
Delta1/2†	31	0.27	2.3 (0–9)	8.3 ± 1.2	25	0.28	8.6 (2–16)	33.9 ± 3.1

* Monoclonal antibodies.
† 5 nm gold was used for immunogold labeling quantification with all the antibodies selective for the glutamate receptor subunits, except for GluR4, which was analyzed using 5 nm and 10 nm.

a general dendritic targeting signal. Since the endoplasmic reticulum is present throughout dendrites (Walton et al., 1991; Terasaki et al., 1994; Krijnse-Locker et al., 1995; Katayama et al., 1996; Spacek and Harris, 1997) and may form a continuous membranous structure, receptors and other dendrite membrane proteins may reach their dendrite location by diffusion through the endoplasmic reticulum, coupled with a local mechanism at different synapses. Selective expression of receptors and other membrane proteins at synaptic populations would be achieved by interactions with proteins at the PSD, which would specifically anchor the receptor (Hunt et al., 1996; reviewed by Kirsch et al., 1996). Such a mechanism for NMDA receptors is supported by the presence of several related proteins, including PSD95/SAP90, SAP97, and Chapsyn-110, which interact with the C-termini of the NR2 subunits (Cho et al., 1992; Kistner et al., 1993; Kornau et al., 1995; Müller et al., 1995; Hunt et al., 1996; Kim et al., 1996). Recently, GRIP was identified as a protein that interacts with the C-terminus of some AMPA receptor subunits (Dong et al., 1997). GRIP interacts with the C-termini of only GluR2 and GluR3 and, therefore, would provide a mechanism for selective anchoring of AMPA receptors based on their subunit compositions. Such proteins could determine both the location and the number of receptor molecules at a synapse. If targeting is achieved in this fashion, receptors associated with intracellular membranes, which presumably are in transport to and from postsynaptic membranes, should be uniformly distributed throughout dendrites, with no relationship to the synaptic location of the receptors. In support of such a mechanism, we have seen GluR4 and mGluR1α associated with intracellular membranes in apical dendrites of fusiform cells (Figures 2 and 4).

Targeting could also be achieved by selective degradation of a protein in a particular domain of the cell. For example, our results could be explained if apical dendrites contain machinery to selectively and rapidly degrade GluR4 and mGluR1α. We are unaware of such a mechanism functioning in neurons. We also cannot rule out the possibility that our results are due to a selective masking of the epitopes at one population of synapses and not the other. The limited availability of antibodies to glutamate receptors prevents testing this possibility with multiple antibodies. However, for mGluR1α, which is selectively present at auditory nerve synapses, two different antibodies were used, and the same results were obtained. Furthermore, the absence of mGluR1α from apical synapses is supported by physiological studies showing that mGluRs modulate parallel fiber-evoked activity on cartwheel neurons but not on fusiform neurons (Molitor and Manis, 1997). It also should be noted that the anti-GluR2/3 antibodies used in this study probably recognize GluR4c (Gallo et al., 1992). Therefore, it is possible that the selective distribution of GluR4 does not include the GluR4c variant.

While the selective location of mGluR1α could be achieved by targeting mRNA or protein, the presence of GluR4 at basal, but not apical, synapses suggests that at least two different AMPA receptor complexes are formed, those with GluR4 and those without. If, for example, targeting is achieved by mRNA, GluR4 could

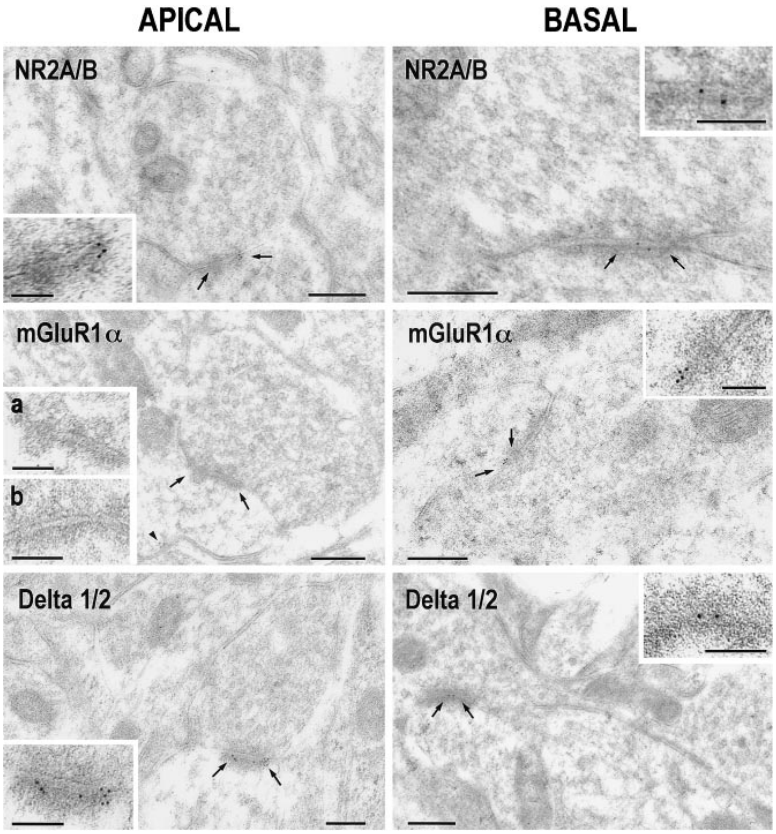

Figure 6. Postembedding Immunogold Labeling with Antibodies Selective for NR2A/B, mGluR1α (Monoclonal Antibody) and Delta 1/2 (5 nm Gold) in Apical and Basal Dendrites of Fusiform Cells

The images on the left show parallel fiber synapses making synaptic contact on dendritic spines (NR2A/B, mGluR1α) and a dendrite shaft (δ 1/2) of apical dendrites of fusiform cells. The postsynaptic membranes at these synapses show immunogold labeling for NR2A/B and δ 1/2 but not for mGluR1α (insets, [a] 5 nm gold, [b] 10 nm gold). On the right, auditory nerve synapses on basal dendrites of fusiform cells have postsynaptic immunogold labeling with all three antibodies. The insets show higher magnifications of the synaptic membranes (small, closed arrows). The small arowhead shows cytoplasmic gold labeling (mGluR1α). Scale bars for NR2A/B, mGluR1α, and δ 1/2, 0.25 μm; for the insets, 0.1 μm.

be synthesized in basal dendrites into a homomeric receptor, and it would be present with complexes made up of GluR2 and GluR3 at auditory nerve synapses. For it to form a complex with GluR2 and GluR3, GluR2 and GluR3 would need to be partially assembled in the cell body, and assembly with GluR4 would be completed in the dendrites. If all three subunits are synthesized in the cell body, the assembly mechanism would need to allow for complexes with and without GluR4. Neurons may indeed synthesize receptor complexes with different subunit combinations; in hippocampal CA1 neurons that express GluR1, 2, and 3, immunoprecipitation studies show that AMPA receptor complexes that contain GluR1/GluR2, GluR2/GluR3, and only GluR1 are present (Wenthold et al., 1996).

The architecture of fusiform cells, with two distinct dendritic branches, would facilitate targeting of receptors by sending different receptors to different branches. Whether or not such targeting also occurs for synapses on the same branch or on a neuronal cell body must be investigated, but existing evidence would support selective targeting. In the hippocampus, the NR1 subunit of the NMDA receptor is far less abundant postsynaptic of mossy fiber terminals on CA3 pyramidal neurons than it is at other synaptic populations (Petralia et al., 1994a; Siegel et al., 1994). In the adult rat cerebellum, δ receptors are present at parallel fiber synapses but not climbing fiber synapses, while AMPA, kainate, NMDA, and metabotropic glutamate receptors are present at both populations (Landsend et al., 1997; Zhao et al., 1997). These populations of synapses are present on the same dendritic branches of Purkinje neurons.

Establishing the Composition of the Postsynaptic Receptor

The presynaptic input is an obvious candidate in considering which factors determine the postsynaptic receptor composition. However, the presynaptic input alone cannot determine the receptor composition in fusiform cells, since cartwheel cells in the superficial DCN also receive synaptic input from parallel fibers but contain mGluR1α at their postsynaptic membranes, along with several other glutamate receptors (Moliter and Manis, 1996; Petralia et al., 1996). Also, synapses between the auditory nerve and neurons in the anteroventral cochlear nucleus differ in their receptor compositions (Hunter et al., 1993). The developmental time course of synapse formation could also be important in determining the postsynaptic receptor composition; for fusiform cells, the characterization of synapse development has not been carried out. Data on the δ receptor in Purkinje neurons suggest that synaptic receptor composition is established as the synapse matures (Lomeli et al., 1993; Mayat et al., 1995). At ten days of age, both parallel fiber and climbing fiber synapses have δ receptors, but in the adult, these receptors are lost from climbing fiber synapses (Landsend et al., 1997; Zhao et al., 1997). Synaptic activity, therefore, may play a critical role in regulating the postsynaptic receptor composition.

Experimental Procedures

Antibodies

The antibodies used in this studies are shown in Table 1. All have been thoroughly characterized and widely used for immunocytochemical localization of glutamate receptors with light microscopy

and both pre- and postembedding immunocytochemistry. Although it has not been demonstrated, both antibodies to GluR2/3 probably recognize GluR4c, a splice variant of GluR4 (Gallo et al., 1992). In cases where two different antibodies to the same receptor were used, which included antibodies to NR1, GluR2/3, and mGluR1α, indistinguishable results were obtained with the two antibodies.

Retrograde Labeling with Horseradish Peroxidase and Tissue Preparation

Sixteen Sprague-Dawley rats were used in this study, 14 for preembedding immunocytochemistry and 4 for freeze-substitution and immunogold labeling. For injection of horseradish peroxidase, 13-day-old rats were anesthetized with a mixture of ketamine HCl (Ketaset; 100 mg/ml; Fort Dodge Laboratories, Inc.) and xylazine (Rompun; 20 mg/ml; Miles, Inc.) at 0.1 ml per 100 g of body weight and were placed in a Stoelting stereotaxic apparatus. A small cut was made in the skin to expose the skull. Using Lambda as the reference point (Paxinos and Watson, 1986), a small hole (1 mm in diameter) was made in the skull, 2 mm caudal and 2 mm lateral. Using a Hamilton syringe, a 5%–10% solution of HRP (Sigma Type VI) was injected by pressure into one inferior colliculus. After injection, animals were kept in an incubator at 37°C until they recovered completely. Animals were then returned to the mother. Two days after the injection of HRP, animals were anesthesized as described above and perfused transcardially. The animals used for preembedding immunocytochemistry were perfused with a fixative consisting of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.12 M phosphate buffer (pH 7.2). After a perfusion time of 5–10 min with 200 ml of fixative, the brains were removed and fixed for an additional hr at 4°C. They were rinsed in three changes of phosphate buffered saline (PBS) and were stored overnight at 4°C in PBS. Coronal and sagittal sections (40–50 μm) of the brain stem were cut in cold PBS with a vibratome (Pelco DTK-3000W microslicer). The animals used for freeze-substitution and immunogold labeling were perfused with a fixative consisting of 4% paraformadehyde and 0.5% glutaraldehyde in 0.12 M phosphate buffer (pH 7.2). After a perfusion time of 5–10 min with 200 ml of fixative, the brains were left intact for 5 hr at 4°C, removed, and fixed in the same liquid fixative for an additional 30 min at 4°C. They were rinsed in three changes of 0.1 M phosphate buffer (pH 7.2) containing 4% glucose and were stored overnight at 4°C in the same buffer. Sagittal sections (150 μm) of the brain stem were cut with a vibratome in cold 0.1 M phosphate buffer (pH 7.2) containing 4% glucose. Agarose in PBS (1%) was usually used to support the brain stems. The use and care of the animals in this study were done following the guidelines of the NIH Animal Research Advisory Committee.

Preembedding Immunocytochemistry

The preembedding immunocytochemical technique for glutamate receptors, as described in detail by Petralia and Wenthold (1992), was used. Briefly, the slices were incubated for 1 hr in 10% normal goat serum in PBS for polyclonal antibodies or in 1.5% normal horse serum for monoclonal antibodies. The slices were incubated in primary antibody (Table 1) in PBS overnight at 4°C and processed using the avidin-biotin-peroxidase system (Vectastain kit, Vector Laboratories, Burlingame, CA). Antibody binding was visualized using 3′,3-diaminobenzidine tetrahydrochloride (DAB) in PBS (10 mg/20 ml) and 5 μl 30% hydrogen peroxide. Sections were routinely preincubated for 10–15 min in DAB to increase penetration. Controls were done by omitting the primary antibody.

Sections used for light microscopy were mounted on slides using 1% gelatin in 25% ethanol. Air-dried slides were placed in xylene, and coverslips were attached with Permount. Sections used for electron microscopy were fixed in 1% osmium tetroxide in PBS for 1 hr, washed in PBS (3×) over 1 hr, dehydrated in an ascending battery of ethanols (50%, 70%, 80%, and 96%, 2 changes of 3 min each for all concentrations; and 100%, 3 changes of 7 min each) and flat-embedded in Poly/BED 812 resin (Polyscience, Inc., Warrington, PA) between polyethylene sheets (ACLAR, Ted Pella, Inc., Reeding, CA). Ultrathin (75–80 nm) semi-serial sections were cut parallel to the plane of section of the tissue, using an Ultracut-S ultramicrotome. In this way, we obtained the entire surface of the nucleus in a single ultrathin section. This procedure was necessary to get a proper orientation for electron microscopy, and was useful for the localization of the dendrite segments of the fusiform cell in the different layers of the DCN. The electron microscope analysis was done on unstained ultrathin sections with a JEOL JEM-100CX II transmission electron microscope at 60 kV.

Freeze Substitution and Immunogold Labeling

The freeze substitution and postembedding immunogold technique for glutamate receptors, as described in detail by Matsubara et al. (1996), was used. Retrogradely transported HRP was developed with DAB in PBS (10 mg/20 ml) and 5 μl 30% hydrogen peroxide for 10 min. The DCNs were carefully dissected from the brain stem slices. Freeze-substitution and low-temperature embedding of the sections in a metacrylate resin were performed (van Lookeren Campagne et al., 1991; Hjelle et al., 1994; Chaudhry et al., 1995). Briefly, the sections were cryoprotected by immersion in graded concentrations of glycerol (10%, 20%, and 30%) in 0.1 M phosphate buffer and plunged rapidly into liquid propane (−184°C) cooled by liquid nitrogen in a Leica EM CPC cryofixaton unit (Reichert, Vienna, Austria). The samples were immersed in 0.5% uranyl acetate dissolved in anhydrous methanol (−90°C, 24 hr) in an AFS cryosubstitution unit (Reichert). The temperature was raised in steps of 4°C/hr from −90°C to −45°C. The samples were washed three times with anhydrous methanol and infiltrated with Lowicryl HM20 resin (Polyscience, Inc., Warrington, PA) at −45°C with a progressive increase in the ratio of resin to methanol. Polymerization was performed with ultraviolet light (360 nm) for 48 hr.

Postembedding Immunocytochemistry

Colloidal gold-coupled goat anti-rabbit IgG (5 nm GAR G5 and 10 nm GAR G10; Amersham) was used to detect rabbit polyclonal antibodies, and goat anti-mouse IgG (5 nm GAM G5, 10 nm GAM G10, and 15 nm GAM G15) was used to detect mouse monoclonal antibodies (Table 1). All procedures were done at room temperature. Ultrathin sections (60–70 nm) on nickel grids (300 mesh) were incubated in the following solutions: 1) 0.1% sodium borohydride and 50 mM glycine in Tris-buffered saline containing 0.1% Triton X-100 (TBST; 10 min); 2) 10% normal goat serum (NGS) in TBST (10 min); 3) polyclonal primary antibodies against GluR2, GluR2/3, GluR4, NR1, NR2A/B, and Delta 1/2 or monoclonal primary antibodies against GluR2/3, NR1, and mGluR1α (Table 1) in TBST containing 10% NGS (2 hr); 4) TBST (10 min); 5) 10% NGS in TBST (10 min); 6) colloidal gold-coupled secondary antibody diluted 1:20 in TBST containing 10% NGS and polyethyleneglycol (5 mg/ml; 1 hr). The ultrathin sections were counterstained with 1% uranyl acetate and 0.3% lead citrate and studied with a JEOL JEM-100CX II transmission electron microscope at 60 kV. Controls were done by omitting the primary antibody.

Double labeling with a polyclonal antibody that recognizes GluR4 (Wenthold et al., 1992) and a monoclonal antibody that recognizes GluR2/3 (Nusser et al., 1994; Ottiger et al., 1995) was done in the same incubation step. Colloidal gold-coupled secondary antibody (5 nm anti-rabbit and 15 nm anti-mouse) diluted 1:20 in TBST containing 10% NGS and polyethyleneglycol (5 mg/ml; 1 hr) were incubated together. Control experiments, in which the rabbit GluR4 antibody was incubated with the anti-mouse secondary antibody, or the mouse GluR2/3 antibody was incubated with the anti-rabbit secondary antibody, showed no immunoreactive labeling.

Quantitative Analysis of Immunogold Labeling

One hundred fifty-five postsynaptic densities of the auditory nerve and one hundred twenty-three postsynaptic densities of the parallel fibers were analyzed (Table 2). Fourteen to thirty-five densities were analyzed for each antibody. Data were collected from cases only where the postsynaptic density was well defined; the length of the postsynaptic densities was measured and the number of associated gold particles was counted. Only gold particles clearly seen at the postsynaptic density and within the synaptic cleft were counted. The maximum distance allowed between the PSD and a gold particle was 14 nm, based on the spatial resolution of the immunogold technique (Merighi and Polak, 1993). All synapses which could be identified as either auditory nerve synapses or parallel fiber synapses (criteria presented in Results) were included in the analysis.

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